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PRINCIPAL INVESTIGATOR: Lisheng Ge, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh  
Pittsburgh, Pennsylvania 15260

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13. ABSTRACT <i>(Maximum 200 words)</i> Our laboratory has recently devised two potential gene therapeutic approaches, the utilization of alternative splicing as a control element in the chimeric enzyme/prodrug therapy (CEPT), and dual modulation vectors. Both approaches aim at increasing the tissue-specific expression of gene therapeutic agents. Based on differences in CD44 variant isoform expression between normal and malignant cancer cells, we postulate that selective killing of primary and metastatic cancers may be achieved by utilizing alternative splicing signals of CD44 variant exons as control elements in CEPT. In our colon cancer metastasis to liver model, we developed dual modulation vectors to increase tissue-specific expression of cytosine deaminase (CD) and protect normal cells. The activity of oppositely inserted albumin promoter is used to downregulate the non-specific expression of CEA promoter in liver cells. We report here our initial efforts to test (1) the activity of chimeric fusion protein CD44/CD in mammalian cells and (2) the activity of dual modulation vector using the CAT report gene.			
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## Introduction

A promising approach called the virus-directed enzyme/prodrug therapy (VDEPT) has been developed for the treatment of primary and metastatic tumors (1). This approach is based on the concept that transcriptional differences between normal and neoplastic cells can be exploited to selectively kill tumor cells. In VDEPT, an artificial chimeric gene is created that is composed of a tissue-specific transcriptional regulatory sequence (TRS) linked to the coding domain of a non-mammalian enzyme. When expressed in eukaryotic cells, the non-mammalian enzyme metabolically activates an administered non-toxic prodrug to a cytotoxic anabolite. In VDEPT, If the TRS is from a tumor-associated gene, then the chimeric gene will produce tumor-specific enzymatic activity and consequently, tumor-specific production of the cytotoxic anabolite. VDEPT therapeutic approaches have been developed for the treatment of hepatocellular and colorectal carcinomas. For hepatocellular carcinoma, the TRS from  $\alpha$ -fetoprotein is used to control the expression of the varicella-zoster virus thymidine kinase (VZV-TK) gene (1), and for colorectal carcinoma, the TRS from carcinoembryonic antigen (CEA) gene is used to control the expression of the Escherichia coli (E.coli) cytosine deaminase (CD) gene (2). Once selectively expressed in the cancer cells, VZV-TK initiates the anabolism of the prodrug, 6-methoxypurine arabinoside (araM), to the toxic compound araATP enabling the killing of hepatoma cells. For colorectal tumor cells metastatic to the liver, CD deaminases the administered non-toxic prodrug 5-fluorocytosine (5-FC) to the metabolically activated and toxic 5-fluorouracil (5-FU). The efficacy of these two enzyme/prodrug systems have been compared in cell lines and animal studies. While 50% of TK-expressing cells are required to achieve a eradication of 60% of tumors, significant antitumor effects were observed with only 4% of the cells expressed CD (3).

Our laboratory has recently developed two novel approaches as potential gene therapy treatment to cancers. In the first approach, we utilize CD44 alternative splicing signals as gene therapeutic targeting control elements. CD44 is a heterogeneous protein that exhibits a variety of variant (CD44v) isoforms. Genomic cloning and DNA sequencing have revealed that the CD44 gene is composed of at least 20 exons, 12 of which can be alternatively spliced (4-6). Primary diversity generated by alternative splicing is mediated at a unique extra cellular site, where up to 10 of the alternatively spliced exons may be inserted in varying combinations into an extra cellular framework of six invariant exons to produce molecules known as CD44 variant isoforms (CD44v). Based on clear differences in the expression of these CD44v isoforms between normal and malignant tissues, we believe that CD44 alternative splicing signals may provide a new gene therapeutic targeting approach in the cancer treatment. This approach involves the substitution of CD44 alternative splice signals for the transcriptional regulatory sequences as the control element in the chimeric enzyme/prodrug therapy (CEPT). If CD44 variant alternative splicing signals are expressed only in tumor cells and not in adjunct parenchymal cells, then only tumor cells capable of CD44 variant expression can direct the chimeric gene to produce tumor-

specific expression of the non-mammalian enzyme and consequently, tumor-specific production of the cytotoxic anabolite.

We have devised another gene therapeutic approach referred to as Dual Modulation Vectors to increase tissue-specific expression of gene therapeutic agents. The dual modulation constructs are constructed to contain tumor tissue-specific transcriptional regulatory sequence upstream of the E.coli CD gene, and another transcriptional regulatory sequence oppositely inserted downstream of CD gene. Competition for transcriptional activity of the two transcriptional regulatory elements functions to target in a tissue-specific manner the activity of the CD "suicide gene". The central premise in construction of the dual modulation vectors is that in tumor metastasis, inclusion of dual transcriptional regulatory elements function to synergistically maximize tissue specific expression of CD "suicide gene" in tumor cells while minimizing potentially hazardous non-specific expression of CD gene in surrounding normal parenchymal cells. In our colon cancer metastatic to liver model, carcinoembryonic antigen (CEA) promoter and albumin promoter are oppositely inserted to flank the E.coli CD gene. When transfected into colon tumor cells, CD converts the administered prodrug 5-FC to the cytotoxic 5-FU, resulting in killing of colon tumor cells. In colon tumor metastasis to liver, the non-specific expression of CD in normal liver cells would be downregulated by antisense CD RNA transcribed by the albumin promoter thereby protecting normal liver cells. Hence our focus on generating control elements that function to protect surrounding parenchymal cells results in effectively increasing the specificity of tumor cell expression. This approach may be also used in other tumor metastasis. Although metastasis of tumors may be widespread, it is common that involvement of a single secondary site is involved. These secondary sites are predictable based on the identity of the primary tumor. For example, prostate cancer is metastasized in bone, and breast cancer in the chest wall, regional nodes or bone.

The objectives of this research are: (1) to test if CD44/CD fusion proteins possess equivalent wild type enzymatic activity in the mammalian cells; (2) to construct dual modulation vectors which exhibit tissue specific expression. We report here our initial efforts to utilize CD44v tumor-mediated alternative splicing signals and dual modulation vectors as additional gene therapeutic targeting control elements.

## Research Design and Methods

### Construction of CD44/CD chimeric vector and cell transfection

The *E.coli* cytosine deaminase gene was cloned by PCR amplification and the enzymatic activity of CD44c(v8v9)CD chimeric fusion protein was tested in bacteria as described previously (7). The CD activity of chimeric CD44c(v8v9)CD was equal to the wild-type CD. To construct mammalian expression vector, the CD gene and CD44c(v8v9)CD constructs were cloned into mammalian expression vector, pBK-RSV (Stratagene), giving pBK-RSV-CD and pBK-RSV-CD44c(v8v9)CD (Fig. 1). Breast cancer cell line BT-20 were grown in Dulbecco's modified eagle medium supplemented with 10% fetal calf serum. The DNA constructs were stably transfected into cells using Lipofectin (GIBCO BRL). After selection under G418, the clones were selected and then used for clonogenic assays. Briefly, cells were plated in 3.5-cm diameter tissue culture wells and grown 7-10 days at 37° C in the presence of 5-FC (0, 250 and 500  $\mu$ g/ml). Cells were then fixed with methanol and stained with Geimsa.

### Construction of dual modulation vectors

Mammalian expression vector pSVK3 (Pharmacia), an SV40 promoter based eukaryotic expression plasmid was selected as the foundation for construction of the dual modulation vectors. The bacterial chloramphenicol acetyltransferase (CAT) report gene was inserted into the multiple cloning sequence site of pSVK3 to make plasmid pSV-CAT. This plasmid is used as reference for promoter activity since the SV40 promoter exhibits transcriptional activity in virtually all human cells or tissues. Subsequently, the SV40 promoter was replaced with a 370-bp human CEA DNA sequence to make plasmid pCEA-CAT. This CEA DNA sequence (-299 to +69) was amplified by PCR from human genomic DNA and confirmed by DNA sequencing (8). Similarly, the 410-bp rat albumin promoter (a gift from Dr. Joseph Locker of University of Pittsburgh) was used to replace SV40 promoter to generate pALB-CAT. For construction of dual promoter constructs, the albumin promoter was inserted oppositely downstream of CAT gene, giving plasmid pCEA-CAT-ALB. To further increase the albumin promoter transcription, we inserted three  $\alpha$ -fetoprotein (AFP) enhancer elements (gift from Dr. Joseph Locker) downstream albumin promoter, resulting in pALB-CAT. These AFP enhancers have been demonstrated to stimulate albumin promoter (9). Finally, in dual modulation constructs, the DNA sequence containing polyadenylation fragment was isolated from the plasmid pBK-RSV (Stratagene) and inserted into a PvuII restriction site, located 5' of the CEA promoter (Fig. 2) to provide transcription termination and polyadenylation of the albumin transcribed mRNA,

### Cell transfection and CAT assay

Colon tumor cell line SW1463 and hepatocellular carcinoma cell line Hep 3B were obtained from the American Type Culture Collection. Cells were grown in

Dulbecco's modified eagle medium supplemented with 10% fetal calf serum. For transient transfection, cells were seeded in 60-mm dishes and grown until about 75% of confluence. The same molar concentration of DNA constructs containing CAT gene were transfected into the cells using Lipofectin. To monitor transfection efficiency, 0.5  $\mu$ g of plasmid pCMV- $\beta$ -gal was co-transfected. After two days, cells were washed twice with PBS and scraped into 1.0 ml of PBS. Cells were pelleted in microcentrifuge tubes at 4° C. The supernatant was discarded and cells were resuspended in 100  $\mu$ l of 0.25 M Tris-HCl. Cell extracts were prepared by three times of freeze-thaws and followed by centrifugation. Protein concentration of cell extracts was determined using BIO-RAD's protein assay. The assay of  $\beta$ -Galactosidase in cell extracts was performed according to Sambrook (10). The transfection efficiency was normalized for all cell extract samples. For CAT assay, Stratagene's FLASH CAT nonradioactive CAT assay 1-deoxyCAM was used. The reaction includes 55  $\mu$ l of cell extracts and reaction buffer, 15  $\mu$ l of BODIPY 1-deoxyCAM substrate reagent and 10  $\mu$ l of 4 mM acetyl CoA. The reaction was incubated at 37° C for 2 hours, and extracted by adding 1.0 ml of ice-cold ethyl acetate. Following centrifugation at 10,000 x g for 3 min. The supernatant was transferred to a new tube and evaporated in a speed vacuum. The yellow residue in the tube was dissolved in 30  $\mu$ l of ethyl acetate. The sample was spotted to the TLC plate. The TLC plate was performed in a TLC tank with 87:13 mixture of chloroform-methanol. For quantitative analysis, reaction products were excised from TLC plates, then 1ml of methanol was added. The samples were Vortexed and centrifuged. Then the supernatant was removed and the absorption was measured at 490 nm using a spectrophotometer.

## Results and Discussion

### Cytotoxicity of tumor cells expressing with CD and chimeric DC44c(v8v9)CD

We have previously demonstrated that enzymatical activity of the chimeric CD44c(v8v9)CD fusion protein exhibits wild-type levels of CD activity in a spectrophotometric assay that measures the deamination of cytosine to uracil in *E.coli* cell lysates (7). A critical factor to the success of this CEPT approach is that the chimeric CD44/CD fusion protein retains sufficient enzymatic activity to generate metabolically cytotoxic anabolites when given 5-FC as substrate. A number of fusion proteins have been previously engineered, most notably diphtheria and cholera toxin-encoding gene derivatives, both of which suffer from toxicity effects when applied to human subjects (11-13). Our strategy to synthesize a CD44/CD chimeric fusion protein ultimately capable in human cells involves incorporation of the five following genetic elements: (1) a 5' leader sequence capable of initiating and directing CD44 translation to the appropriate subcellular location; (2) fusion of this sequence in translational reading frame with the appropriate CD44 exons present in genomic splicing constructs; (3) fusion of the CD44 exons again in translational reading frame with cytosine deaminase gene; (4) mutation of the *E.coli* cytosine deaminase f-Met initiation codon from GTG to ATG to facilitate transcription in eukaryotic cells; and (5) appropriate 3' SV40 sequences for termination

and polyadenylation to be contributed by the mammalian expression vector. We showed here that the chimeric CD44c(v8v9)/CD DNA construct transfected breast cell line BT-20 exhibited markedly reduced survival in 5-FC. There is also a clear dose-response relationship between 5-FC concentration and killing of cells (Fig. 3).

In our colorectal cancer metastatic to liver model, we rely on tumor-specific expression of cytosine deaminase to be regulated by the promoter element of CEA. CEA is a tumor associated marker which is expressed in a large percentage of primary and metastatic colorectal carcinomas (CRC) and not in liver. CEA expression is used routinely as an important diagnostic tool for postoperative surveillance, monitoring of chemotherapy efficacy, immunolocalization, and immunotherapy (14-16). Although expression of CEA is virtually linked in adulthood to the development of CRC, transcriptional control of various reported CEA promoter elements may show significant activity in CEA(-) cells. A recent investigation identified several important regions of the 5'-flanking sequences of CEA that demonstrated high selectivity and strong expression in CEA-positive cells. However, these DNA regions still exhibit expression in varying degrees in CEA-negative liver cells (8). A second and complementary approach would be to construct vectors that contain not only the CEA promoter responsible for promoting tissue-specific expression, but inclusion of transcriptional regulatory sequences that would function to downregulate CEA expression in the liver cells. Such a system may be approached by inserting downstream of the CD "suicide gene" liver specific transcriptional regulatory sequences that function to downregulate CD expression through production of antisense CD mRNA or competition with host cellular transcription factors thereby limiting initiation of CEA promoter elements. We have devised the dual modulation vectors as a potential gene therapeutic approach in colon tumor metastasis to liver. The strategy in this application is to only express cytosine deaminase in colon tumor cells and protect parenchymal liver cell surrounding the cancerous cells. We used the albumin promoter as the second promoter in the dual modulation vectors. The albumin promoter region has been shown to be a tissue-specific promoter (17-18). A 410-bp rat albumin promoter was inserted oppositely in the dual modulation vector. To test the dual modulation constructs, The CAT report gene was inserted between CEA promoter and albumin promoter.

Two cell lines were used to test the dual modulation vectors, one is colorectal cancer cell line SW1463, and another hepatocellular carcinoma cell line Hep3B. We first tested the activity of DNA constructs with single promoters in transiently transfected SW1463 cells and Hep3B cells. In the colon cell line SW 1463, CEA promoter exhibits much stronger activity than that of SV40 promoter, albumin promoter, and albumin promoter plus three AFP enhancers (Fig. 4). In the hepatocellular cell line Hep3B, the albumin promoter shows stronger activity than that of CEA promoter and SV40 promoter, and the albumin promoter plus the AFP enhancers exhibit lower activity than the albumin promoter alone. These results reveal that (1) the substantial non-cell specific transcription can occur for both CEA promoter and albumin promoter and (2) as expected, AFP enhancers substantially increase the activity of albumin promoter in liver cells (Fig. 5).

We further tested the dual modulation construct, pCEA-CAT-ALB, in liver cells. The CAT activity from pCEA-ACT-ALB reduced by 50% compared to the activity of pCEA-CAT (Fig. 6). This result demonstrated that the albumin promoter in the dual modulation constructs could downregulate CEA promoter transcription activity. To achieve more efficient downregulation of CEA promoter activity, we inserted three AFP enhancers downstream of albumin promoter, making plasmid pCEA-CAT-ALBe. Unexpectedly, the AFP enhancers increase not only the activity of albumin promoter in liver cells, but also the activity of CEA promoter in liver cells (data not shown). To prove if the AFP enhancers are tissue-specific, we constructed plasmid containing the CAT gene under the control of SV40 promoter plus the AFP enhancers. Cell transfection studies demonstrated that the AFP enhancers also increase the activity of SV40 promoter in liver cells (data not shown). It appears that the AFP enhancers are liver cells specific enhancers, not promoter specific.

## Conclusion

We report here our initial efforts to develop two potential gene therapeutic approaches. Both approaches are devised to increase tissue-specific expression of CD "suicide gene". In the chimeric enzyme/prodrug therapy (CEPT), we are interested in incorporating the molecular control elements of alternative splicing of CD44 variants into the CEPT approach to direct tumor-restricted CD expression. The initial results demonstrated that CD44/CD chimeric fusion proteins possess sufficient enzymatic activity to make viable chemotherapeutic agents. Further experiments will include construction of CD44 genomic DNA constructs containing the alternatively spliced CD44 variant exons, e.g. CD44g(v8v9), and testing of the splicing of these genomic CD44 variant exons in tumor cells. If these genomic CD44 variant exons successfully splice in the tumor cells, these CD44 variant exons will be used in CEPT for animal study.

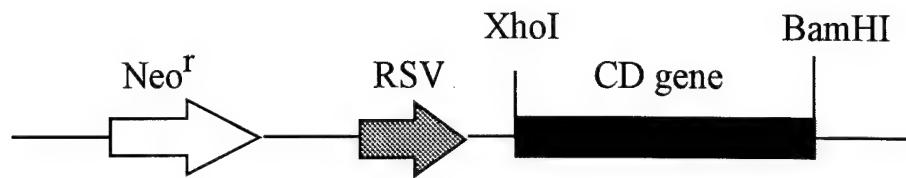
For the dual modulation vectors, we report here our preliminary studies. We have demonstrated that both CEA promoter and albumin promoter show significant activity in non-tissue specific expression. This non-specific expression would result in killing of normal cells if these transcriptional regulatory sequences are used in CEPT. We also demonstrated that in dual modulation vectors, the albumin promoter could reduce the CEA activity up to 50%. We discovered that the AFP enhancers also stimulate the CEA promoter in liver cells. Therefore, either a stronger liver cell-specific transcriptional regulatory sequence or an albumin-specific enhancer is needed to downregulate more efficiently, if not completely, the non-specific expression of CEA promoter in liver cells. The albumin gene enhancer, which is located 10 kb upstream, may be a good candidate to be used to increase the transcription efficiency of the albumin promoter (17).

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pBK-RSV-CD



pBK-RSV-CD44/CD

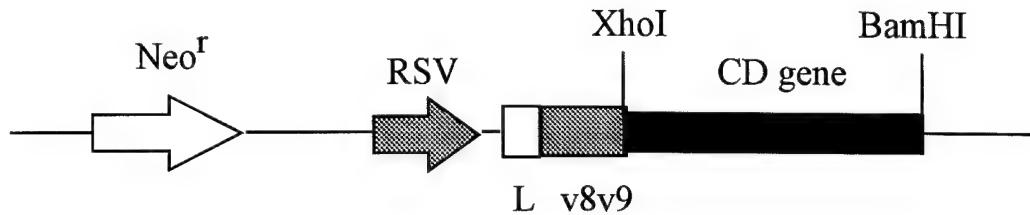


Figure 1. Structure of the cytosine deaminase (CD) and chimeric DC44/CD cDNA. L, CD44 leader and secretory peptide sequence; v8v9, cDNA of CD44 alternatively spliced exon v8 and exon v9.

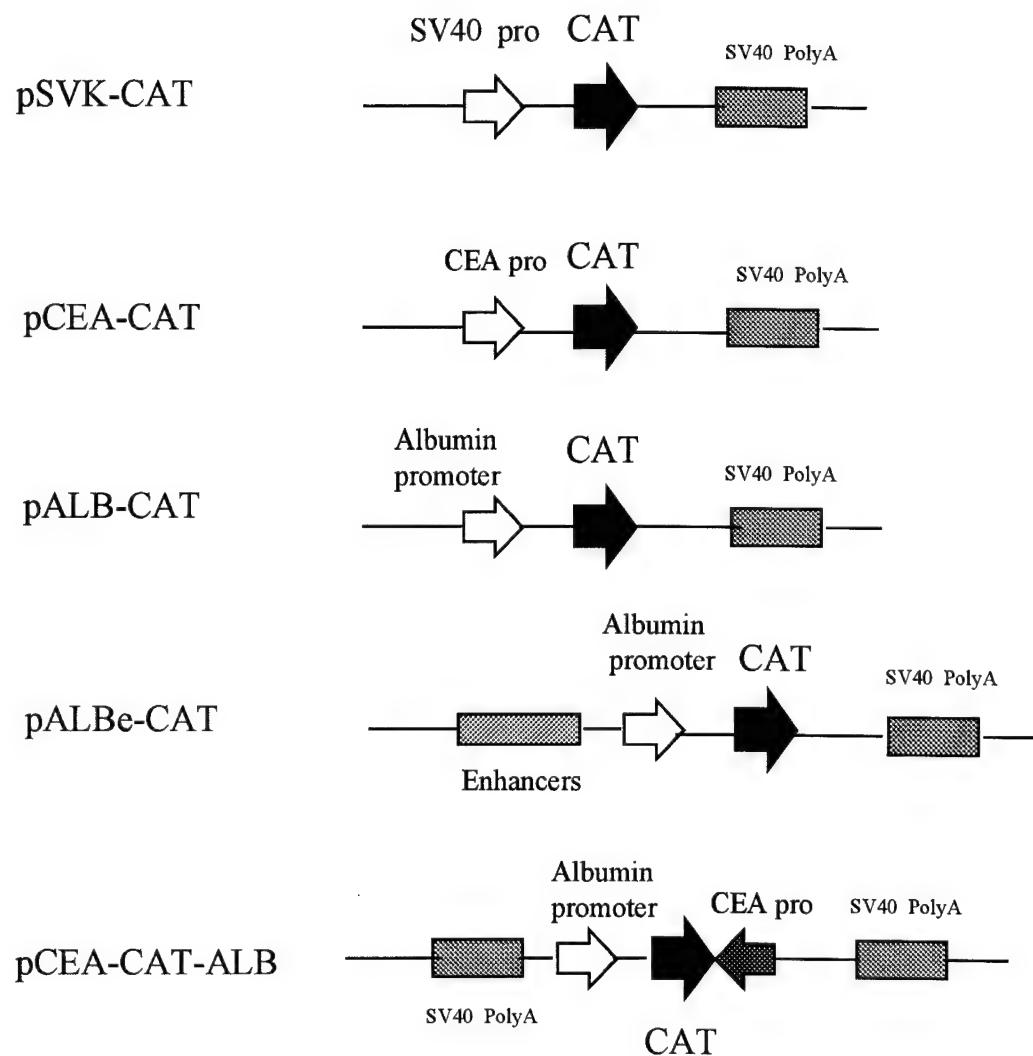
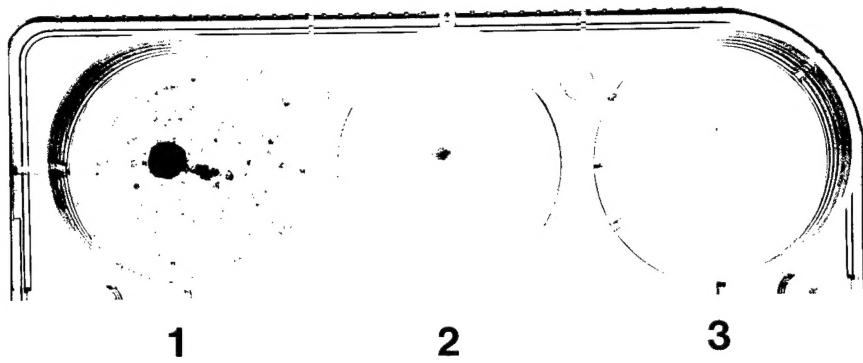


Figure 2. DNA constructs containing the single promoters, SV40, CEA and albumin; and dual promoters, CEA and albumin. CAT gene is used as report gene.

A.



B.

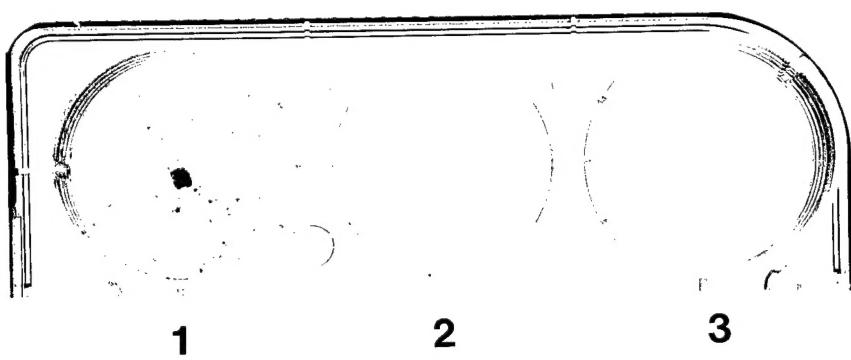


Figure 3. Clonogenic Assays. A. Breast cancer cell line BT-20 was transfected with pBK-RSV-CD. B. BT-20 was transfected with pBK-RSV-CD44c(v8v9)CD. Plate No. 1, 2 and 3 represent 5-FC concentration 0, 250  $\mu$ g/ml, 500  $\mu$ g/ml, repectively.

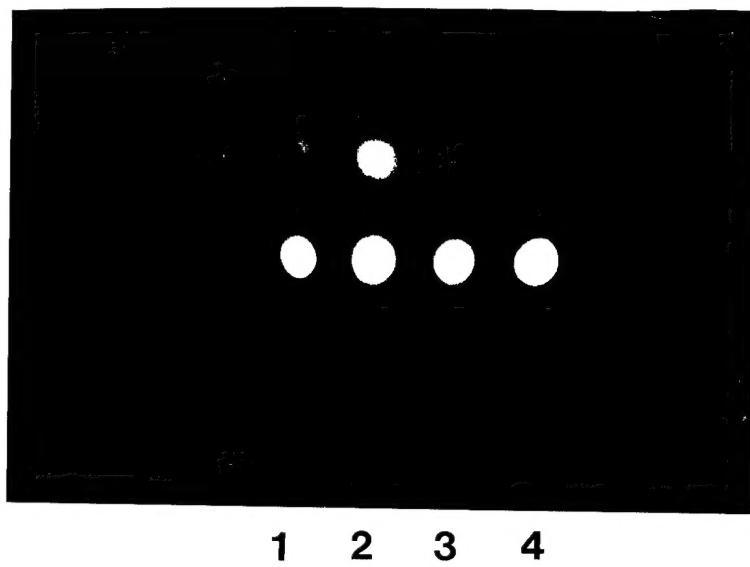


Figure 4. CAT activities of DNA constructs containing CAT gene in colon cancer cell line SW1463. 1. pSV-CAT; 2. pCEA-CAT; 3. pALB-CAT; 4. pALBe-ACT.

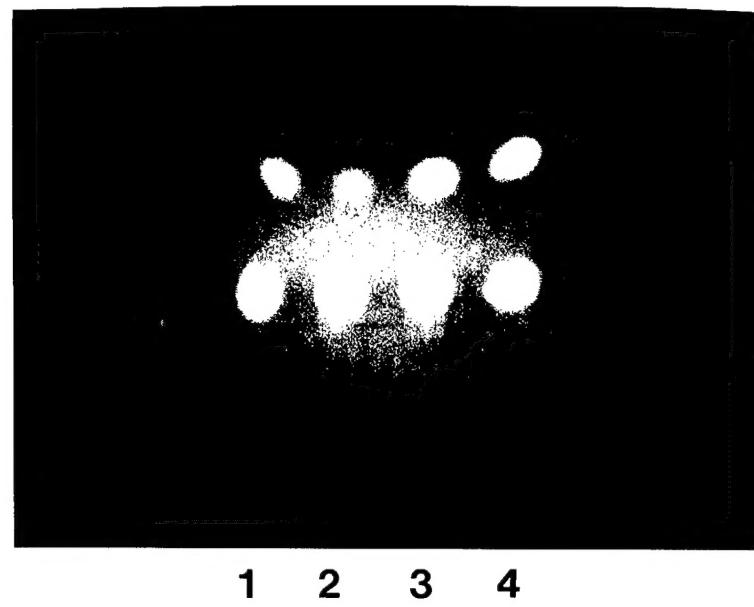


Figure 5. CAT activities of DNA constructs containing CAT gene in hepatocellular carcinoma cell line Hep3B. 1. PSV-CAT; 2.pCEA-CAT; 3. PALB-CAT; 4. PALBe-CAT

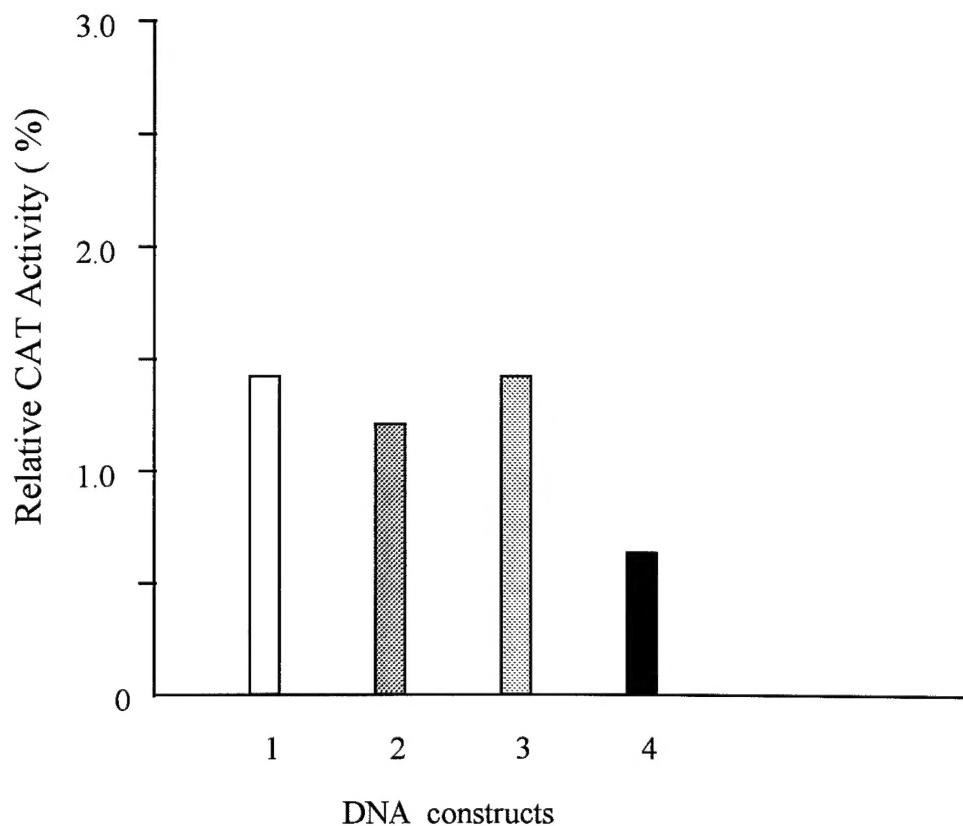


Figure 6. Comparison of promoter activities in Hep3B cells. 1. pSVK-CAT, 2. pCEA-CAT, 3. pALB-CAT, 4. pCEA-CAT-ALB.